

A genomic approach to colon cancer risk stratification yields biologic insights into therapeutic opportunities

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Gene expression profiles provide an opportunity to dissect the heterogeneity of solid tumors, including colon cancer, to improve prognosis and predict response to therapies. Bayesian binary regression methods were used to generate a signature of disease recurrence in patients with resected early stage colon cancer validated in an independent cohort. A 50-gene signature was developed that effectively distinguished early stage colon cancer patients with a low or high risk of disease recurrence. RT-PCR analysis of the 50-gene signature validated 9 of the top 10 differentially expressed genes. When applied to two independent validation cohorts of 55 and 73 patients, the 50-gene model accurately predicted recurrence. Standard Kaplan–Meier survival analysis confirmed the prognostic accuracy ($P < 0.01$, log rank), as did multivariate Cox proportional hazard models. We tested potential targeted therapeutic options for patients at high risk for disease recurrence and found a clinically important relationship between sensitivity to celecoxib, LY-294002 (PI3kinase inhibitor), retinol, and sulindac in colon cancer cell lines expressing the poor prognostic phenotype ($P < 0.01$, t test), which performed better than standard chemotherapy (5-FU and oxaliplatin). We present a genomic strategy in early stage colon cancer to identify patients at highest risk of recurrence. An ability to move beyond current staging by refining the estimation of prognosis in early stage colon cancer also has implications for individualized therapy.

biology | personalized medicine | progression

Colorectal cancer is the second leading cause of cancer death in the United States today. In 2008, in the United States alone, it is estimated that 148,810 people will be newly diagnosed with colorectal cancer, making this an important cause of morbidity and mortality. Of newly diagnosed cases, $\approx 40\%$ are diagnosed while the cancer is in an early or localized stage (1).

The genetic description of colorectal cancer, as first detailed in the classic studies of Vogelstein and Fearon, and Vogelstein *et al.* (2, 3), laid the foundation for the concept of accumulation of genetic alterations as tumors progress to a malignant state. These studies emphasized the concept of heterogeneity, an idea more fully described in recent studies that comprehensively monitored the cancer genome for genomic imbalances and associated gene expression changes (4, 5). Genome-wide expression analysis thus offers the opportunity to characterize and treat tumors in an individualized fashion.

Since the initial description by Wood (6), the clinical staging system in colon cancer has been the standard for determining prognosis. Developing improved prognostic tools is important as the current array of clinical predictors provides only broad categorizations of risk and insufficiently characterizes the relative risk for recurrence in individual patients (7). As an example, patients with early stage colon cancer (stages I and II) are usually considered cured after surgical resection, despite the fact that 15–20% of these patients develop disease recurrence (8, 9). In breast and lung cancer

(10–12), genomic approaches have been shown to direct the care of cancer patients. We hypothesize that an improved understanding of patterns of gene expression in individual patients could lead to better, more directed care of patients with early stage colon cancer.

Results

Gene Expression Signature of Recurrence in Early Stage Colon Cancer.

There is a significant unmet need to further characterize and treat early stage colonic tumors in an individualized fashion. This is particularly relevant for patients diagnosed with early stage colon cancer (stages I and II) who are usually considered cured after surgical resection, despite the fact that up to 20% of these patients later develop disease recurrence (8, 9). Because the current tumor, node, metastasis (TNM) staging system is relatively imprecise, our aim was to develop a prognostic model using gene-expression data to predict disease recurrence after curative surgery (a clinically relevant phenotype) in early-stage colon cancer. Toward this end, as detailed in Fig. 1A, we developed a prognostic model using a collection of 52 samples representing clinical stage I and stage II disease, for which gene expression data were available. Two independent datasets of 55 and 73 samples were used for validation of the prognostic model. The clinical characteristics of the patients are detailed in supporting information (SI) Table S1.

Using Bayesian regression methods (12–14), we identified gene expression profiles, or metagenes, constituted by 50 genes that predict the risk of recurrence in an initial ‘training’ cohort of 52 patients with early-stage colon cancer (Fig. 1B Top). The predictive accuracy of the 50-gene model was assessed by using leave-one-out-cross-validation in which the analysis is performed repeatedly with one sample removed each time and the probability of recurrence is predicted for that sample. The 50-gene model predicted recurrence with an accuracy of 90.3% (Fig. S1) with a significant difference in the predicted probability of recurrence or ‘recurrence scores’ between the two groups ($P < 0.001$) (Fig. 1B Middle). Furthermore, a receiver operator characteristic (ROC) analysis revealed that the area under the curve was 0.94 ($P < 0.0001$) and established the optimal cut-point for the Recurrence Score at 0.76

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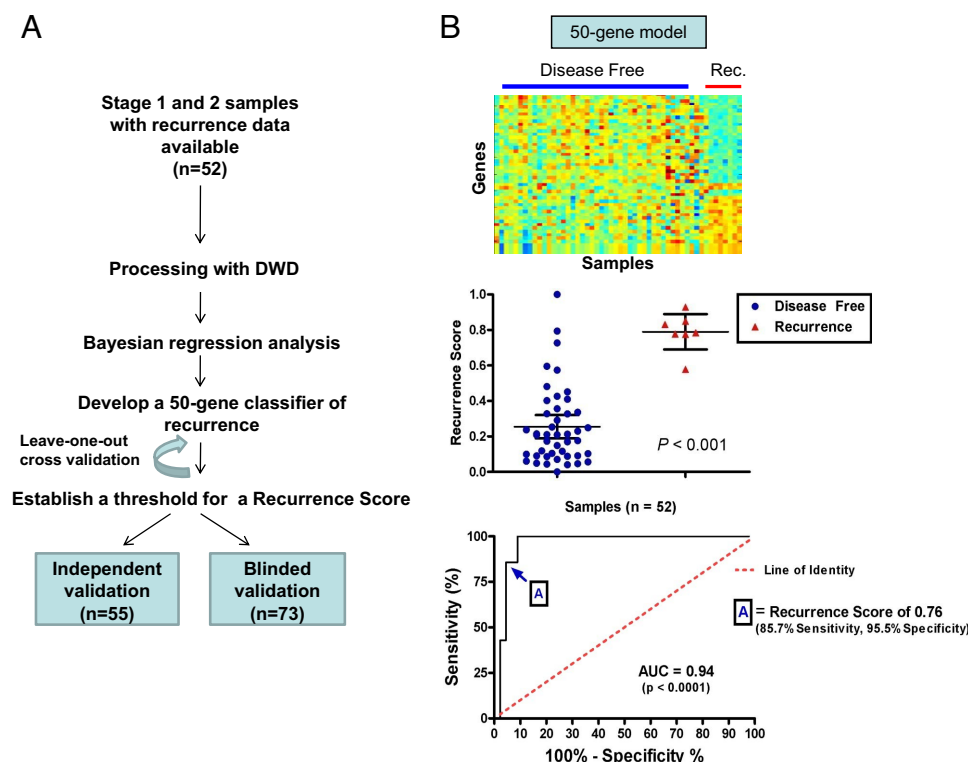


Fig. 1. Development of 50-gene predictor of Recurrence. (A) Consort diagram. To develop a genomic predictor of colon cancer recurrence, we made use of a training cohort of 52 patient samples representing clinical Stage 1 and Stage 2, for which gene expression data were available to develop a prognostic model. Two independent data-sets of 55 and 73 samples were used as validation cohorts. (B) Development of the 50-Gene Predictor of Recurrence. (Top) Shown are the heat-map of the samples used to develop the metagene model (the training set) with blue and red representing extremes of expression with visually apparent differences in gene expression. Samples from patients who remained disease free are on the left ($n = 43$), and samples from patients who had disease recurrence are on the right ($n = 7$). (Middle) Shown is a comparison individual and mean Recurrence Scores, or the probability of recurrence as predicted by the 50-gene model, by group as compared by t test ($P < 0.001$). (Bottom) Shown is the ROC curve identifying the Recurrence Score of 0.76 as the optimal cut-point to be used to classify samples in the validation set. The area under the curve (AUC) of 0.94 further confirms the robustness of the 50-gene model.

(Sensitivity: 85.71%, Specificity: 95.56%, likelihood ratio: 19.29) (Fig. 1B Bottom). Identification of the genes in the 50-gene model revealed that many were genes known to play a role in carcinogenesis (RAS family and TNF family) and metastasis (GRK6, GAS6, CLAPIN1, zinc finger proteins, and ubiquitin pathway genes) (Table S2). Finally and importantly, the prognostic ability of the 50-gene model was similar irrespective of TNM stage in early stage colon cancer (Fig. S2).

As an additional measure of validity in predicting colon cancer recurrence, we tested the accuracy of the metagene model in an independent dataset of patients with stage I/II colon cancer that was recently made available (15) (Table S1). In this cohort of 55 patients all followed for a minimum of 5 years post-resection, our model of recurrence correctly predicted 38/55 (69.1%) samples (using the predetermined cut-point); the mean recurrence score in the disease-free cohort was significantly different ($P = 0.002$, t test) than the mean recurrence score in those with disease recurrence (Fig. 2A).

To further confirm the prognostic capability of the metagene model, we applied the 50-gene model to an independent validation dataset comprising 73 colon cancer patients treated at the University Medical Center Göttingen, Germany (GSE10402), using the predetermined Recurrence Score cut-point of 0.76. Importantly, the outcome was blinded to the investigator (C.R.A.) performing the analysis. In this independent blinded validation analysis, nearly all of the patients with recurrence were predicted by the model to recur resulting in a sensitivity of 90% (Fig. 2B). In a Kaplan–Meier survival analysis (Fig. 2C), statistically significant differences were seen between the cohorts predicted to recur (high recurrence score) and those predicted to be disease-free (low recurrence score). Importantly, almost all those predicted by the model to remain disease-free (low recurrence score) did so with only one case of clinical recurrence in that group (negative predictive value: 97%). Although the overall accuracy was lower than anticipated (61%), this likely reflects the inclusion of patients in this cohort with more advanced disease (stage III) and fewer than three years of follow up; it is possible that some of these individuals will in fact recur if followed for a longer time period (5 years).

In addition, RT-PCR validation of the top 10 differentially expressed candidate genes (*CCL24*, *FNI*, *GAS6*, *PARD6B*, *HNT*, *PARVB*, *POSTN*, *SLC2A5*, *TNFRSF1B*, and *TRIM36*) demonstrated that 9 of 10 genes (all except *HNT*) identified to be most differentially expressed in our genomic model could also be validated by using RT-PCR (Fig. 3).

Finally, as confirmation that the 50-gene model is independently prognostic in early stage colon cancer, we performed univariate and multivariate analyses by using a Cox proportional hazards model. As seen in Table 1, a prediction of recurrence (based on the 50-gene model) was an independent poor prognostic variable in both univariate and multivariate analyses ($P = 0.01$). These results demonstrate that the 50-gene model of disease recurrence has prognostic implications independent of traditional prognostic criteria such as age, gender, and stage of disease (tumor size and lymph node status).

Recurrence Signature Identifies Therapeutic Opportunities. The primary goal of improved prognosis, and in particular, the ability to identify patients at high risk for recurrence, is the capacity to identify those patients in need of more effective therapy. Although others have used gene expression data to predict prognosis in colon cancer, none have validated their models in a robust manner nor have any linked the gene expression data to therapeutic strategies (16–19). Using gene expression methodologies to understand the molecular mechanisms involved in cancer progression may be helpful beyond prognosis because this knowledge may lead to the study of drugs that target relevant, deregulated pathways in an individual patient. More importantly, we may be able to identify specific, effective agents from a repertoire of currently existing drugs.

One source of information to guide this strategy is the Connectivity Map, a project developed at the Broad Institute, to assemble a reference collection of gene-expression profiles from cells that have been treated with a variety of drugs. This effort established links between gene expression profiles and drugs (20). We queried the Connectivity Map to identify drugs that might be connected to

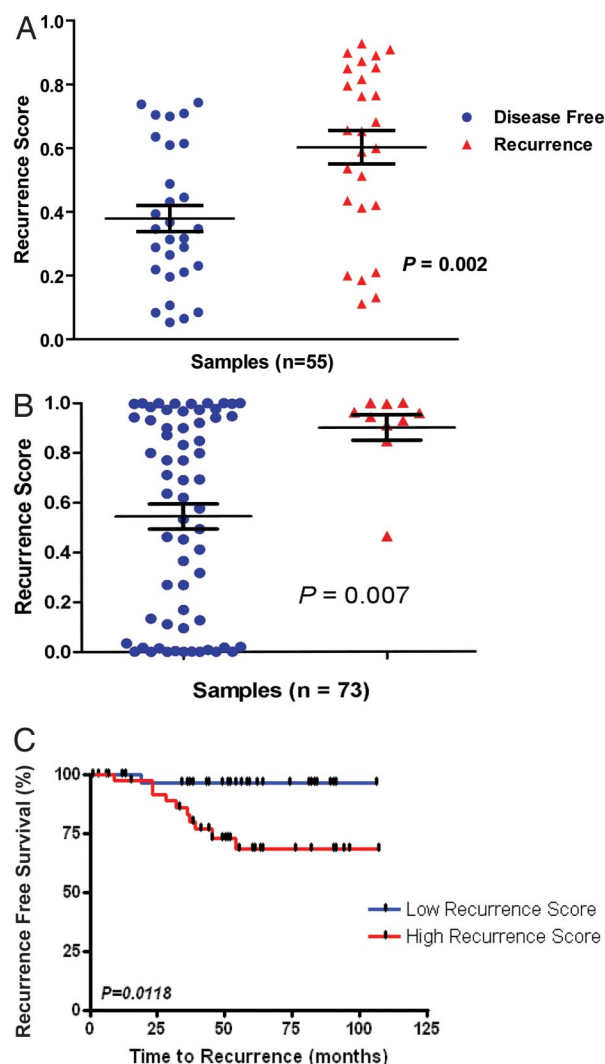


Fig. 2. Validation of the 50-gene predictor of colon cancer recurrence. (A) Independent validation of the prognostic model: the scatter plot compares individual and mean Recurrence Scores for the MEXP-1224 cohort ($n = 55$) grouped by actual recurrence ($P = 0.002$, t test). (B) Blinded validation of the prognostic model: the scatter plot demonstrates a comparison of mean Recurrence Scores for the cohort ($n = 73$) grouped by actual recurrence ($P = 0.007$, t test; 90% sensitivity). (C) The Kaplan-Meier survival analysis demonstrates time to recurrence for the two groups: the blue curve represents those patients predicted to remain disease-free by the model, and the red curve represents those predicted to have recurrence.

the 50-gene colon cancer recurrence signature (genes listed in Table S2, drugs identified by the Connectivity Map in Fig. S3). Four candidate drugs identified by this approach included Tretinoin (a retinol analog), the PI3K inhibitor LY-294002, sulindac, and celecoxib. Interestingly, COX2 inhibitors like celecoxib have been repeatedly identified in the literature as potential agents for reversing polyp growth, particularly in familial adenomatous polyposis syndromes (21–23), although their role in prevention of colon cancer recurrence has not been explored.

Linking Gene Expression with Therapeutic Opportunities in Colon Cancer. To evaluate the potential therapeutic efficacy of the candidate agents (Retinol, LY-294002, sulindac, and celecoxib) identified by using the connectivity map analysis, we mapped the Recurrence Score (using the 50-gene model) to a collection of 14 colon cancer cell lines so as to classify the cell-lines as representative

of the high-recurrence risk phenotype (with high Recurrence Scores) or the low-recurrence risk phenotype (with low Recurrence Scores). Fig. S4 shows the individual cell lines classified by Recurrence Scores. Fig. 4A Left shows the stratification of the cell lines by recurrence score and their respective mutational events [KRAS, p53, BRAF, PI3K, CTNNB2 (beta-catenin), APC and CDKN2A] [http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=study&study_id=4 (accessed Sept 9, 2008)], demonstrating that the risk categories determined by our model of recurrence do not segregate based on any one mutation and simulate the genetic heterogeneity seen in clinical practice.

We hypothesized that the cell lines with high Recurrence Scores would be more sensitive to the candidate drugs (i.e., celecoxib, LY294002, retinol, and sulindac) than cell lines with low Recurrence Scores. We further predicted that these candidate agents could reverse the high risk phenotype by changing the pattern of gene expression. In vitro cell proliferation assays, colon cancer cell lines were treated with celecoxib, LY294002, retinol (used as a surrogate for tretinoin), and sulindac. The clinically relevant controls for these experiments were cytotoxic agents currently used in the treatment of colon cancer, i.e., 5-fluorouracil and oxaliplatin (24). Biologically relevant differences in drug sensitivity (between cell lines with high and low Recurrence Scores) were observed for three of the candidate agents: celecoxib ($P = 0.01$), LY294002 ($P = 0.008$), and retinol ($P = 0.01$) (Fig. 4A). In comparison, traditional chemotherapy agents (5-FU and oxaliplatin) did not show a significantly greater predilection for inhibiting growth in the cell lines with a high Recurrence Score. Linear regression analyses of the probability of recurrence phenotype/recurrence scores and sensitivity to an individual therapeutic agent in vitro (Fig. S5) revealed a significant correlation for COX2 (celecoxib) ($P = 0.03$) and PI3kinase inhibition (LY294002) ($P = 0.02$), suggesting that specific COX2 and PI3Kinase inhibitors could be valuable as initial agents in therapeutic intervention studies. Thus, celecoxib and LY294002 were chosen for follow-up experiments to evaluate the therapeutic potential of these agents to reverse the ‘high risk’ phenotype; again, 5-FU and oxaliplatin were used as controls.

In an effort to simulate high risk phenotype reversal in vitro with celecoxib and LY294002, we used colon cancer cell lines (HCT15, HT29, WIDR, DLD-1, HCT116, and COLO-320) that exhibited high Recurrence Scores (Fig. S3). As shown in Fig. 4B, in multiple replicate experiments, treatment with LY294002 and celecoxib resulted in a significant reduction in the expression of the high recurrence phenotype as shown by the decrease in Recurrence Score ($P = 0.002$, ANOVA). In comparison, the cell line experiments using 5-FU and oxaliplatin failed to show a significant reduction ($P = 0.19$) in the Recurrence Score after treatment (Fig. 4B Right and C). Although most of the cell lines did not demonstrate a significant reduction in Recurrence Score after exposure to traditional chemotherapy (Fig. 4C), two cell lines had a marked reduction in Recurrence Score after exposure to the traditional chemotherapy; this inconsistency across cell lines likely depicts the heterogeneity of response to 5-FU and oxaliplatin seen in actual clinical practice and highlights the need for a more a rational approach to therapy.

Discussion

The ability to understand biological complexity is frequently limited by the lack of precision in defining clinically relevant phenotypes. This is perhaps most relevant for cancers where the oncogenic process, involving the somatic acquisition of a myriad of mutations, coupled with variability within the genetic composition of the host, produces a disease of vast complexity. To develop effective therapeutic strategies, an understanding of the unique characteristics of the individual tumor is critically important. Conventional methods of characterizing tumors rely primarily on visual information including tumor size, degree of metastasis, and histological tumor characterization. Gene expression profiles, which represent biolog-

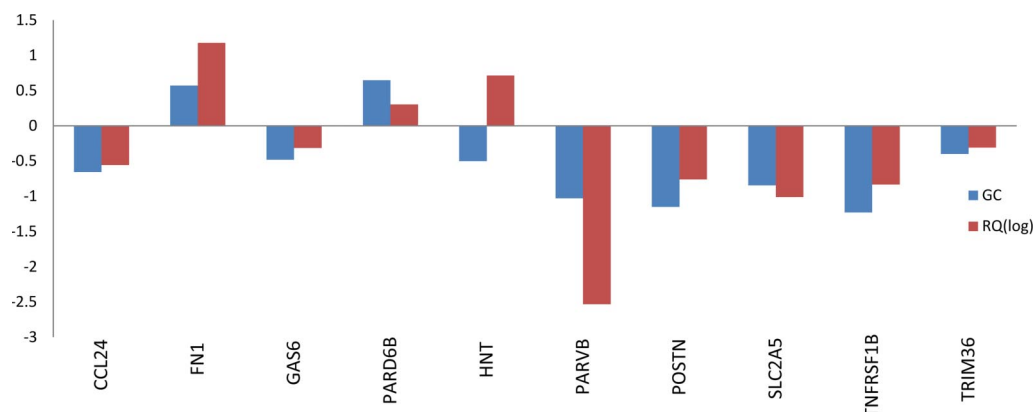


Fig. 3. RT-PCR validation. In vitro RT-PCR assay of the top 10 differentially expressed genes demonstrates concordance in 9 of 10 genes between the PCR results and the 50-gene microarray-based signature. Data are presented as a comparison between the gene coefficients (specific to each gene in the Bayesian model) of the candidate genes and the log of the RQ values for the respective genes in the RT-PCR experiments.

ical states in the form of a pattern of gene expression, offer the opportunity to characterize and treat tumors in an individualized fashion.

Currently, robust strategies for identifying appropriate patients for adjuvant chemotherapy in early stage (stage II) colon cancer are lacking. Although 5-fluorouracil and oxaliplatin represent the standard of care for metastatic disease, their role in the adjuvant setting of early stage disease is less clear. Further, our data also suggest that the tumor response to 5-fluorouracil and oxaliplatin is variable. The primary questions that need to be addressed are, which stage II colon cancer patients need to be treated, and how each patient should be treated. In this article, we propose a general strategy for predicting disease recurrence. In the clinically relevant instance of early stage disease, a gene expression signature predictive of disease recurrence can suggest effective therapeutic strategies (COX2 and PI3Kinase inhibition) for individuals with the high-risk gene expression phenotype. Evidence for the efficacy of this approach is provided by applying these therapeutic agents *in vitro* and demonstrating the reversal of the high-risk phenotype in cell lines, in a manner more specific and more effective than standard chemotherapy (5-fluorouracil or oxaliplatin).

Using Bayesian regression analyses, we identified a 50-gene model that accurately predicts recurrence in patients with early stage colon cancer. Importantly, this classifier was validated in an independent dataset in a blinded fashion. The ability to identify a poor prognostic group within those with early stage disease emphasizes the need to identify effective therapeutic strategies for that group. Using in vitro cell proliferation assays, we demonstrated a clinically relevant relationship between sensitivity to certain therapeutic agents and a high-risk gene expression phenotype in colon cancer. Celecoxib, LY-294002 (PI3Kinase inhibitor), retinol, and sulindac may all serve as potential therapeutic agents in the

treatment of stage II colon cancer. The strong correlation between sensitivity to COX2 (celecoxib) and PI3Kinase inhibition (LY-294002) in tumors expressing the high risk phenotype (Fig. S5), and the ability to reverse the profile representative of the high risk phenotype in cell lines (Figs. 4 B and C) illustrate the robustness of this approach.

Although we propose further assessment of the role of celecoxib and PI3Kinase inhibition in the setting of stage II colon cancer, combination therapy may not be the most effective strategy (25–27). Prospective evaluation of an individualized treatment strategy in randomized clinical trials is warranted (Fig. 5).

Applications of gene expression signatures to treatment continue to move in the direction of personalized therapy that is best suited for an individual patient based on tumor phenotype (28, 29). The present study illustrates significant advances in this area by using expression data as a means for not only identifying patients with early stage colon cancer at high risk for recurrence, but also for providing an efficient strategy for selecting appropriate therapies. Importantly, the strategies here will need to be tested in prospective phase III clinical trials, perhaps following the strategy outlined in Fig. 5, to confirm the validity of the approach and to further assess the efficacy of drugs/agents proposed to be effective in these patients. Such a study is now being planned. This refined strategy has the potential to change the current paradigm for surveillance and treatment in colon cancer.

Methods

Patient Samples and Data. From publicly available gene expression data collections, all early stage colon cancer patients (stages I and II) with known survival outcomes were identified to constitute the initial training dataset ($n = 52$) for the development of a genomic predictor of disease recurrence. Two independent

Table 1. Univariate and multivariate Cox proportional hazard model demonstrating the independent prognostic ability of the 50-gene predictor of recurrence in early stage colon carcinoma

Factor	Hazard ratio	Lower, 95% C.I.	Upper, 95% C.I.	P value
Univariate Cox Proportional Hazard Analysis				
Age (continuous)	0.99	0.94	1.04	0.65
Gender (male/female)	0.85	0.22	3.3	0.81
Stage of disease (III/II)	3.65	0.78	17.21	0.07
Genomic prediction of recurrence	8.29	1.05	65.49	0.01
Multivariate Cox Proportional Hazard Analysis*				
Stage of disease (III/II)	3.18	0.67	15	0.10
Genomic prediction of recurrence	7.54	2.95	59.69	0.01

*Only significant factors (univariate $P < 0.1$) were included in the multivariate analysis.

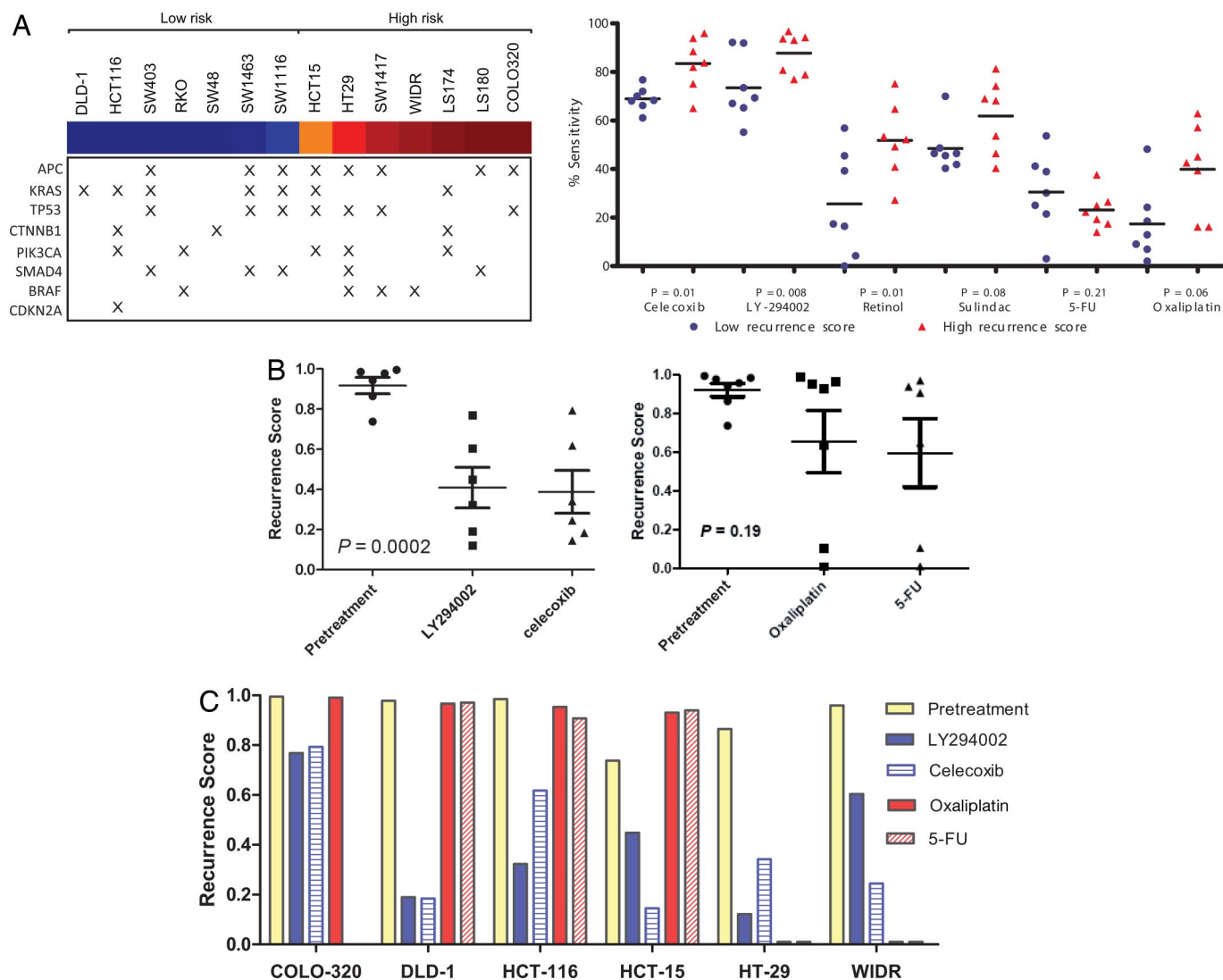


Fig. 4. In vitro validation of candidate drug sensitivity. (A) The left panel shows the mutational events seen in the cohort of colon cancer cell lines, sorted based on the probability of recurrence based on the 50-gene model (blue: lowest risk score, red: highest risk score). (Right) Shown are the results of experiments performed using 14 colon cancer cell lines. Gene expression data from these cancer cell lines was used to classify them according to Recurrence Scores (blue: low risk, red: high risk), using the 50-gene model. The cell lines were then treated with specific drugs identified as candidate agents by using a connectivity map analysis of the 50-gene model. For each drug, sensitivity as measured by cell death using cell proliferation assays is shown in the figure and compared between groups. The cell lines with high Recurrence Scores appear to be more sensitive to drug than those with low Recurrence Scores, demonstrating that treatment modalities involving the use of celecoxib, LY294002 (PI3Kinase inhibitor) and retinol may be beneficial in patients with colon cancer who are at high risk for disease recurrence. (B) (Left) Shown is the change in Recurrence Score after exposure to PI3Kinase (LY294002) and COX2 inhibition (celecoxib). An ANOVA analysis demonstrates a significant difference between pretreatment and post treatment (with LY294002, celecoxib) recurrence scores in colon cancer cell lines. (Right) Traditional chemotherapy agents (5-FU and oxaliplatin) do not show a significantly greater predilection for inhibiting growth in the cell lines with a high recurrence score. (C) A histogram shows that all of the cell lines demonstrate a decrease in recurrence score post treatment, indicating a reversal of the high-risk phenotype after exposure to LY294002 or celecoxib, with DLD-1 showing the greatest sensitivity to reversal and COLO-320 showing the least effect. In comparison, the effect of 5-FU and oxaliplatin is inconsistent across the cell lines.

datasets: an Affymetrix dataset ($n = 55$ E-MEXP-1224), representing patients with primarily stage II colon cancer and another plasmid blinded dataset ($n = 73$, GSE10402) representing consecutive patients with early stage colon cancer treated at the University Medical Center Göttingen, Germany, were used to independently validate the 50-gene predictor. Table S1 describes the demographic features of the training and validation cohorts.

Metagene Predictor of Recurrence. To develop a metagene predictor for colon cancer recurrence in early stage disease, a training dataset was created by using samples from stage I and II colon cancer that were linked with clinical outcomes (GSE5206 and GSE2138) ($n = 52$). These datasets were merged by using the Distance-Weighted Discrimination (DWD) (30) (<https://genome.unc.edu/pubsup/dwd/>) method to eliminate any systematic biases. The merged dataset was filtered, and 91 genes with significant recurrence effects ($P < 0.001$) were selected

for further analyses. Using Bayesian binary regression methodologies previously described (12, 13), a metagene predictor of recurrence was developed. A probit function enabled us to generate a probability of recurrence for each sample, referred to as the "Score." An optimal threshold recurrence score value of 0.76 was chosen based on a receiver operating characteristic (ROC) analysis, and was used as the predefined "cut-point", to dichotomize samples into low risk (Recurrence Score < 0.76) and high risk (Recurrence Score ≥ 0.76). The ability of the metagene model was investigated in the two independent datasets in a blinded fashion. For the dataset ($n = 73$) with available time to relapse, standard Kaplan-Meier curves and their significance levels (log-rank test) were generated by using GraphPad Prism version 4.03 for Windows (GraphPad). Univariate and multivariate analyses were performed by using Cox proportional hazard models, and P values reported are based on likelihood ratio tests, and analyses are performed by using the statistical package R (31). See *SI Methods* for complete details.

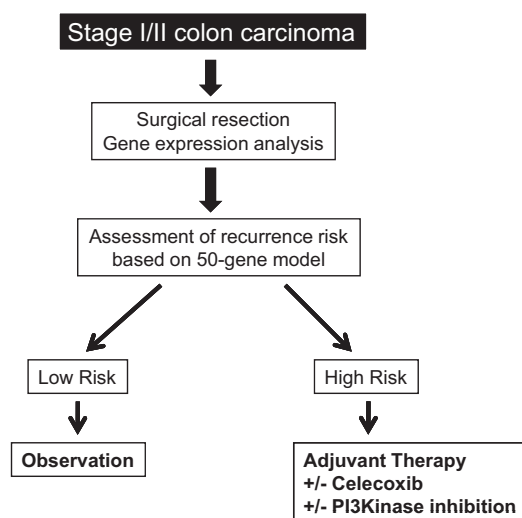


Fig. 5. Clinical application of the 50-gene predictor of early-stage colon cancer recurrence. The schema of a proposed clinical trial that would further validate the prognostic ability of the 50-gene predictor in patients with stage II colon cancer, first identifying low risk patients and then those with high recurrence scores receive adjuvant chemoprevention.

Colon Cancer Cell Lines. To classify cell lines, we measured genome-wide expression in the 14 colon cancer cell lines available through the ATCC, using the Affymetrix U133A Plus 2.0 GeneChip. Complete details of methods involved in growth of the colon cancer cell lines and the in vitro drug sensitivity assays are

available in the *SI Methods*. Total RNA was extracted from the cells with RNeasy kits (Qiagen). The RNA quality was assessed with the use of a bioanalyzer (Agilent 2100 model). Hybridization targets were prepared from the total RNA according to standard Affymetrix protocols. 'Recurrence Scores' were generated for the cell lines and the predefined (from the training set) threshold value was then used to dichotomize the cell lines into low and high risk phenotypes. In vitro cell proliferation assays were used to demonstrate the mean percentage cell death when the highest concentration of drug (celecoxib, retinol, LY294002, sulindac, 5-fluorouracil, and oxaliplatin) was used in each cell line as the basis for comparisons of sensitivity, between high and low recurrence risk groups, for each of the drugs tested. Finally, in the cell lines with high Recurrence Scores, after treatment with targeted drugs, gene expression profiles were reassessed to determine if the high-risk phenotype had been reversed.

Real-Time RT-PCR Validation. The top 10 differentially expressed genes from among the 50-gene model were chosen for further validation by using real-time PCR. Briefly, the methods involved Taqman (Applied Biosystems) custom arrays. Total RNA extracted from each of the seven high risk and seven low risk cell lines are reverse transcribed into cDNA by using random primers. For PCR, Taqman (Applied Biosystems) gene expression assays (including 18s used as the manufacturing control) were used and run on the 7900HT Fast Real-Time PCR System with the Low Density Array Block (Applied Biosystems). After PCR, gene targets were analyzed by assessing Ct values after normalization to GAPDH to compare quantitative expression values.

Further details on patient selection, RNA extraction, preprocessing of gene expression data, and statistical analysis are available as *SI Methods*.

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